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# Crohn's Disease With Latent Tuberculosis Infection or Intestinal Tuberculosis: Rapid Discrimination by Targeted Next-Generation Sequencing

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## ABSTRACT

**Background:** Discriminating Crohn's disease (CD) with latent tuberculosis infection (LTBI) from intestinal tuberculosis (ITB) in tuberculosis-endemic regions remains challenging.

**Aim:** To assess whether targeted next-generation sequencing (tNGS) could be an efficient method for ITB diagnosis and discrimination from CD with LTBI.

**Methods:** The study was conducted prospectively from September 2020 until December 2023. We recruited patients with undetermined intestinal ulcers and positive interferon-gamma release assay. We compared tNGS (using fresh biopsy tissue samples from ulcer bases) to pathological detection of caseous necrotising granuloma, acid-fast bacillus (AFB) staining, tuberculosis polymerase chain reaction (TB-PCR) for diagnostic efficiency. ITB was diagnosed based on cure by anti-tuberculosis therapy and comprehensive clinical evaluation.

**Results:** Of the 100 patients included, 66 had ITB and 34 had CD with LTBI. The sensitivity, specificity, positive predictive value and negative predictive value of tNGS for ITB were 83% (72%–91%), 100% (87%–100%), 100% (92%–100%) and 76% (60%–87%), respectively. TNGS had significantly higher diagnostic sensitivity than AFB staining [15% (4%–39%), $p < 0.05$], TB-PCR [22% (12%–36%), $p < 0.05$] and detection of caseous necrotising granulomas [17% (9%–28%), $p < 0.05$]. The models combining multiclinical factors increased sensitivity (97% vs. 83%) than tNGS alone. No patients with CD and LTBI had positive tNGS.

**Conclusions:** TNGS using fresh biopsy tissue from ulcer bases is highly sensitive and specific, with superiority over other traditional diagnostic methods for ITB detection. TNGS could facilitate rapid diagnosis of ITB and discrimination from CD with LTBI, particularly in high TB-endemic countries.

Lingna Ye and Yushu Cao contributed equally to this work.

## 1 | Introduction

Tuberculosis (TB) is a long-known infectious disease caused by *Mycobacterium tuberculosis* (MTB). Extrapulmonary TB occurs in 20% of all TB cases, and 10% of extrapulmonary TB cases are intestinal tuberculosis (ITB) [1]. TB remains a major health problem in the world, especially in developing countries such as China [2]. Unlike pulmonary tuberculosis (PTB), ITB is difficult to diagnose, with a misdiagnosis rate of 50%–70% in TB-endemic countries [3]. Particularly, differentiating ITB from the ileocolonic form of Crohn's disease (CD) with latent tuberculosis infection (LTBI) that only manifests as intestinal luminal ulcers is very difficult in TB-endemic regions. Misdiagnosis or delayed diagnosis of ITB may result in delayed initiation of anti-tuberculosis therapy (ATT), increasing the risk of mortality [2, 4, 5].

Diagnosis of CD with LTBI must require excluding ITB. The early and rapid detection of ITB in patients with undetermined intestinal ulcers and without manifestation of extraintestinal TB remains a challenge in TB-endemic regions. The Interferon Gamma Release Assay (IGRA) is widely performed for ITB diagnosis. Tuberculosis-specific antigens are used to detect TB effector T lymphocytes in the patient's body [6]. However, patients with active TB cannot be differentiated from patients with either LTBI or a prior history of TB solely based on the IGRA, indicating a limited diagnostic value for active ITB in clinical practice. Pathological detection of caseous necrotising granuloma in biopsied intestinal tissues is one of the key criteria for the diagnosis of ITB and also a good index for the discrimination of ITB from CD. However, detecting caseous necrotising granuloma has very low sensitivity (usually as low as 20%) [7]. In addition, tuberculin acid-fast bacillus (AFB) and tuberculosis polymerase chain reaction (TB-PCR) are used to confirm the clinical diagnosis of ITB [8]. Although the specificity of AFB is as high as 95%, its sensitivity varies from 17% to 70%, while especially low sensitivity is observed for isolated ITB [1, 8]. The specificity of TB-PCR is nearly 90%; however, the pooled sensitivity of PCR was only 44% in a recent meta-analysis of nine studies [8].

It is imperative to develop a highly sensitive and specific method to diagnose ITB and differentiate it from CD with LTBI. Metagenomic next-generation sequencing (mNGS), a high-throughput sequencing technology for microbial DNA, has recently been used in the diagnosis of TB with a good diagnostic value [9–11].

Previous mNGS studies mainly focused on the diagnosis of systemic TB, PTB and tuberculosis meningitis using various specimens such as blood, bronchoalveolar lavage fluid, sputum, lung biopsy tissue and cerebrospinal fluid [10–12]. Compared to mNGS, targeted next-generation sequencing (tNGS), targeting a set of genes related to specific disease phenotypes with high sequencing coverage, only costs about 100 US dollars (approximately 1/3 the cost of mNGS) and has also been used in the diagnosis of TB with high sensitivity and specificity [13, 14].

This study aimed to evaluate the diagnostic performance of tNGS utilising fresh biopsy tissue obtained through colonoscopy. In addition, tNGS was compared to conventional diagnostic methods, including pathological detection of caseous necrotising granuloma, TB-PCR and AFB, in order to examine whether tNGS could be effectively used for the rapid diagnosis of ITB and its discrimination from CD with LTBI in clinical practice.

## 2 | Methods

### 2.1 | Ethics Statement and Informed Consent

This study followed the Declaration of China about the ethical principles for research involving human specimens. All patients or their legal representatives provided written informed consent for the prospective review protocol. The study was approved by the Ethics Committee of Sir Run Run Shaw Hospital in China (Approval number: 20230617) and is registered in the Chinese Clinical Trial Registry (ChiCTR2400088403). Considering the better accessibility and availability of paraffin-embedded (FFPE) biopsy tissue compared with fresh biopsy tissue, we performed preliminary assessment using formalin-fixed, FFPE biopsy samples ahead of fresh samples. The preliminary experiment, included in the whole research design, was approved.

### 2.2 | Participants and Sample Collection

According to inclusion and exclusion criteria, consecutive patients were prospectively recruited in Sir Run Run Shaw Hospital from September 2020 to December 2023. The patients were followed up for 1 year to confirm the final diagnosis.

Inclusion criteria were: (1) age between 18 and 65 years; (2) positive IGRA (T-SPOT.TB kit produced by UK Oxford Immunotec Company) result; (3) undetermined ulcers from the right colon to the end of the ileum with or without ulcers in the other intestinal segments on colonoscopy. Exclusion criteria were: (1) female patients with pregnancy, lactation or plans for future pregnancy; (2) malignancies; (3) severe liver or kidney dysfunction; (4) unwillingness to undergo colonoscopy evaluation; (5) radiological active PTB before colonoscopy; (6) diagnosis of intestinal diseases other than CD with LTBI and ITB.

Briefly, three fresh specimens targeting ulcer bases were obtained by biopsy for direct tNGS detection, while another six to eight specimens from both the same ulcer bases and ulcer margins were obtained for pathological analysis. Following AFB staining, sample sections were examined microscopically. Paraffin blocks with granulomas as large as possible were selected for DNA extraction and PCR according to the manuals of kits. For formalin-fixed, paraffin-embedded (FFPE) biopsy samples that were used for tNGS in preliminary experiments, we also chose granulomas from the ulcer surface for sampling.

### 2.3 | Diagnostics and Outcome Measures

At baseline, every patient was comprehensively evaluated by chest and full abdominal CT scans and colonoscopy. All patients included in the current study were followed up for 1 year. The final clinical diagnosis was based on a comprehensive assessment of clinical manifestations, endoscopic and histopathological characteristics, ATT efficacy and other laboratory

parameters. Extrapulmonary TB was fully evaluated, for example, if the patient had neurological symptoms or ascites, and cerebrospinal fluid examinations, including aetiology, were added. Extrapulmonary TB was defined as TB that involved organs other than the lungs, for example, pleura, lymph nodes, peritoneum and liver. The diagnosis of ITB was made when one of the following criteria was met: (1) positive AFB staining/tNGS/PCR/caseous necrotising granuloma and cure by ATT without subsequent recurrence; (2) clinical, radiologic and colonoscopic evidence of ITB without evidence of other intestinal diseases and cure by ATT without subsequent recurrence.

Colonoscopies were performed at baseline, 8 weeks and 1 year for all recruited cases. For patients with ITB, mucosal healing was defined as the absence of ulcer or active inflammation through colonoscopy. Endoscopic non-response was defined as mucosal ulcerated area reduced by less than half, unchanged or aggravated, compared to baseline. Clinical remission, mucosal healing and no recurrence during 1 year of follow-up indicated cured ITB. All the patients diagnosed with ITB were treated with ATT for 1 year according to local guidelines. The diagnosis of CD was based on the current Chinese guidelines and confirmed when ITB was ruled out with non-response to empirical ATT for 8 weeks. Afterward, CD with LTBI was treated with recommended therapies and simultaneously 4 months of anti-TB prophylaxis with isoniazid and rifampicin if initiation of immunosuppressive therapy and evaluated by colonoscopy at the end of 1 year. Mucosal healing was defined as ulcer-free with the simple endoscopic score for Crohn's disease (SES-CD) ulcerated surface subscore of 0. Endoscopic remission was defined as an SES-CD subscore $\leq 4$ and at least a 2-point reduction from baseline. Endoscopic response was defined as a decrease in SES-CD $> 50\%$ from baseline. Clinical remission was defined as Crohn's Disease Activity Index $< 150$.

### 2.4 | Real-Time Fluorescent Quantitative Polymerase Chain Reaction (RT-PCR)

FFPE biopsy samples were used for RT-PCR. RT-PCR was performed using a commercial TB-PCR kit on an RT-PCR machine (National instrument registration number: 20213401040). According to the manufacturer's instructions, each reaction was performed in 20 μL, comprising 12.5 μL of master mix, 4 μL of double distilled water and 3.5 μL of template. PCR conditions were as follows: 3 min at 95°C, followed by 45 cycles of 15 s at 94°C and 35 s at 60°C. The positive and negative controls included in the kit were used for quality control and reference standards.

### 2.5 | tNGS

The tNGS procedure involved several steps, including sample processing, DNA extraction, DNA fragmentation, adapter ligation, target enrichment, sequencing and bioinformatic analysis. (1) For sample processing, three fresh biopsy tissue specimens were collected per included patient for sequencing. Soybean-sized tissue blocks were collected according to standard sample collection procedures and stored in sterile tubes filled with 2 mL of normal saline, sealed and placed in a foam box with ice packs and immediately transported to the laboratory for pathogen testing. Tissue blocks were cut with scissors and homogenised in 500 μL of normal saline. Then, each sample was broken with 0.5 g glass grinding beads and 50 μL of 20% SDS Lysis Buffer (KS121-WSWTQ-48, KINGCREATE Biotechnology, Guangzhou, China) and homogenised with a high-efficiency tissue cell sample processor (AD1000, ABclonal Technology, Wuhan, China) by centrifugation at 4500 *g* for 45 s at intervals of 20 s and repeated three times for each condition. (2) DNA extraction used a MataPureTM DNA&RNA Extraction Kit (KS121-WSWTO-48, KINGCREATE BIOTECH, Guangzhou, China). (3) In DNA fragmentation, nucleic acid fragments associated with pathogens were gathered through multiplex polymerase chain reaction (multiplex PCR) for library construction and purification with the Library Preparation Kit (KS620-FZGJ96, KINGCREATE BIOTECH, Guangzhou, China). (4) For adapter ligation, the qubit dsDNA HS Assay Kit (Thermo Fisher Scientific Inc.) and Agilent 2100 Bioanalyzer were used for library quality control. Quality control included assessment of DNA concentration, fragment size distribution and library quality using the bioanalyzer or qPCR. (5) For target enrichment, qualified libraries were pooled with equal amounts and sequenced on Illumina Miniseq Dx-CN. (6) For sequencing and bioinformatic analysis, sequencing data were obtained after targeted capture for bioinformatic analysis. By comparing and identifying the obtained sequences with the pathogen sequences in the database, a comprehensive test report was generated.

For FFPEs in the preliminary trial, sample processing was slightly different from that in the formal trial and is listed below. The waxed roll (thickness not exceeding 20 μm, 2–4 sheets; preferably 4 μm thick, 8–10 sheets) was placed into a 1.5 mL EP tube containing 900 μL of buffer DPS. After mixing, the samples were incubated at 56°C with shaking at 400 rpm for 10 min. After centrifugation at 15,000 *g* for 2 min, the resulting pellet was resuspended with 750 μL of ATL buffer and 20 μL of proteinase K, followed by incubation at 56°C with shaking at 600 rpm for 1 h and another incubation at 90°C for 45 min without shaking. Once the system cools to room temperature, differential lysis was performed for human source identification and nucleic acid extraction using magnetic beads. The remaining steps were similar between the preliminary and formal trials.

### 2.6 | Statistical Analysis

Baseline characteristics are shown as mean ± standard deviation or number (%). Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were presented with 95% confidence intervals (CIs). Categorical variables were compared by the Chi-square test or Fisher's exact test. Parametric and non-parametric variables were analysed by the *t* test and the Mann–Whitney *U* test, respectively. Statistical analysis was performed using the R software (version 4.3.0). The detection efficiency was compared between tNGS and other diagnostic methods (AFB staining, TB-PCR and detection of caseous necrotising granuloma) by the McNemar-Bowker $\chi^2$ test. Receiver operating characteristic (ROC) curve analysis was used for correlation analysis between clinical data and tNGS detection, as well as to evaluate the diagnostic performance of tNGS. Areas under the curves (AUCs) with 95% CIs were further calculated. In univariate regression analysis, factors calculated with $p$ values $< 0.05$ were included in the final

prediction model after assessing the collinearity. R package "logistf" was applied for firth logistic model establishment. Statistical significance was set at $p < 0.05$.

## 3 | Results

### 3.1 | Clinical Characteristics of the Participants

As shown in Figure 1, of the 100 finally included cases, 66 were diagnosed as definite ITB and 34 as CD with LTBI (CD-LTBI). All 100 cases were initially treated with ATT for 8 weeks. ITB patients who received ATT had mucosal healing rates of 93.9% and 100% after 8 weeks and 1 year, respectively. All ITB patients had TB in no other organ except for intraperitoneal lymph nodes. The CD-LTBI patients had no endoscopic response to ATT for 8 weeks and were treated as CD cases according to Chinese guidelines. Among 34 CD-LTBI cases, 6 were treated with steroids and thiopurine, 3 with mesalazine, 10 with infliximab, 6 with adalimumab and 9 with Ustekinumab. All of them achieved clinical remission, and none of them had active TB during the 1 year follow-up. Of the 34 CD-LTBI patients, except one underwent terminal ileal and ileocecal resection, 50% of patients showed mucosal healing of the terminal ileum and colon, 30% had endoscopic remission and 20% showed the endoscopic response at the 1 year colonoscopic evaluation.

Using the final diagnosis for categorisation, higher amounts of inflammatory markers, including C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and platelet count (PLT), were detected in the ITB group versus the CD-LTBI group (Table 1). Performing biopsies at the ulcer bases produced no complications, for example, perforation or bleeding.

### 3.2 | Comparison of tNGS for ITB With the Detection of Caseous Necrotising Granuloma, AFB and TB-PCR

All 66 cases of ITB and 34 cases of CD-LTBI underwent tNGS. TNGS detected MTB in 55 of the 66 ITB cases. As shown in Table 2, using the final diagnosis as ITB confirmation, the sensitivity, specificity, PPV and NPV of tNGS for ITB were 83% (72%–91%), 100% (87%–100%), 100% (92%–100%) and 76% (60%–87%), respectively. TNGS had significantly higher diagnostic sensitivity [83% (72%–91%)] than AFB [15% (4%–39%), $p < 0.05$], TB-PCR [22% (12%–36%), $p < 0.05$] and detection of caseous necrotising granuloma detection [17% (9%–28%), $p < 0.05$]. The specificity of tNGS, AFB, TB-PCR and detection of caseous necrotising granuloma was 100%. As shown in Figure 2, tNGS had the best performance among the four diagnostic methods with an AUC of 0.92 (0.87–0.96). All 34 patients with CD-LTBI had negative results for tNGS, caseous necrotising granuloma detection, AFB and TB-PCR. We collected multiple positive findings for the diagnosis of ITB and found that most positive findings from the other three methods have been already covered by tNGS, except one for AFB and one for caseous necrotising granuloma (Table S1). We calculated the diagnostic performance combining tNGS with the other three methods and found that the negative predictive value of tNGS could be slightly improved (79% vs.76%).

### 3.3 | Correlation Analysis Between tNGS-Based Detection of ITB and Clinical Characteristics

Except for CRP, ESR and PLT, tNGS detection rate showed no significant correlations with the factors listed in Table 1. Endoscopic, pathological and radiological characteristics, including old PTB lesions, large granuloma (> 400 μm), right-hemicolonic multiple ulcers (> 4), confined right-hemicolonic lesions, specific shapes of ulcers (circular/insect-eaten) and circular enhancement of lymph nodes and calcification (Table S2) were also included in the analysis. As shown in Figure S1, old PTB lesions, large granuloma (> 400 μm) and right-hemicolonic multiple ulcers (> 4) were positively correlated with NGS-positive results ($p < 0.05$). The other three parameters listed in Table S2 had no significant correlations with tNGS-based detection of ITB.

### 3.4 | Logistic Models Combining tNGS and Other Parameters for the Prediction of ITB Diagnosis

Using the characteristics listed in Table 1 and Table S2, we also tried to develop a model combining the characteristics and tNGS for the final diagnosis (ITB or CD-LTBI). We first performed

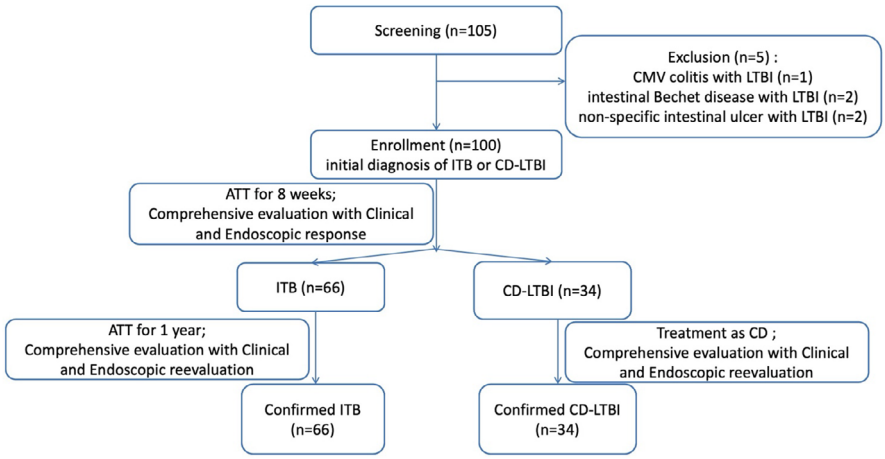


**FIGURE 1** | Flow diagram of ITB diagnosis. ATT, anti-tuberculosis therapy; CD, Crohn's disease; CMV, cytomegalovirus; ITB, intestinal tuberculosis; LTBI, latent tuberculosis infection.

univariate regression for these clinical indicators at first and screened those with $p$ values $<0.05$. The results of univariate regression analysis are presented in Table S3. Factors including CRP, ESR, PLT and large granuloma ($>400\mu m$) showed $p$ value $<0.05$. High collinearity was observed among CRP, ESR and PLT (coefficient $>0.8$). Considering the event per variable (EPV) principle and clinical experience, only CRP was included in the final logistic model. Since perfect separation was observed in tNGS and the other three methods, as well as the small sample size, we applied firth's logistic regression to reduce small sample bias and allow reliable estimation in the presence of separation. Model 1 was the basic model including tNGS, CRP and large granuloma. Considering the additional positive results provided by AFB and caseous necrotising granuloma shown in Table S1, we added the two factors respectively into Model 2 and Model 3. Model 4 combined both factors. Finally, we compared five indicators of these four models, including AUC, sensitivity, specificity, PPV and NPV (Table 3). Adding the other two diagnostic methods did not significantly improve the performance of the basic model.

**TABLE 1** | Baseline clinical characteristics of all included patients.

| | ITB ($n=66$) | CD-LTBI ($n=34$) | $p$ |
|---|---|---|---|
| Characteristic | | | |
| Age, years | 38.0 ± 12.7 | 43.0 ± 15.2 | 0.11 |
| Smoking | 4 (6.1) | 2 (5.9) | 0.99 |
| Sex | | | 0.07 |
| Male *n* (%) | 30 (45.5) | 22 (64.7) | |
| Female *n* (%) | 36 (55.6) | 12 (35.3) | |
| Laboratory parameters | | | |
| WBC ($10^9$/L) | 6.5 ± 3.1 | 6.5 ± 4.9 | 0.99 |
| CRP (mg/M) | 23.9 ± 35.8 | 6.9 ± 10.0 | 0.01 |
| HB (g/L) | 117.0 ± 30.3 | 125.8 ± 26.7 | 0.17 |
| PLT ($10^9$/L) | 283.5 ± 106.3 | 235.5 ± 78.1 | 0.03 |
| ALB (g/L) | 38.2 ± 10.0 | 40.6 ± 9.0 | 0.27 |
| ESR | 30.0 ± 29.4 | 16.3 ± 18.3 | 0.02 |
| Presenting symptoms | | | |
| Diarrhoea *n* (%) | 27 (40.9) | 19 (55.9) | 0.13 |
| Constipation *n* (%) | 4 (6.1) | 1 (2.9) | 0.49 |
| Weight loss *n* (%) | 12 (18.2) | 2 (5.9) | 0.09 |
| Hematochezia *n* (%) | 4 (6.1) | 1 (2.9) | 0.49 |
| Night sweat *n* (%) | 4 (6.1) | 0 | 0.30 |
| Fever *n* (%) | 10 (15.2) | 1 (2.9) | 0.06 |
| Abdominal pain *n* (%) | 48 (72.7) | 22 (64.7) | 0.44 |

*Note:* Data are mean ± standard deviation or number (%). Weight loss was considered from onset to the initial visit (baseline).
Abbreviations: ALB, albumin; CD-LTBI, Crohn's disease with latent tuberculosis; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HB, haemoglobin; ITB, intestinal tuberculosis; PLT, platelet; WBC, white blood cell.

### 3.5 | Preliminary Experiment Using FFPE Biopsy Specimens by Colonoscopy for tNGS Detection of ITB

In the original experimental design, FFPE colonoscopy-biopsied specimens were used for tNGS, as these specimens are much more accessible and convenient for storage than fresh biopsied specimens. Fifteen specimens from 15 patients with definite ITB from our previous database were sent for sequencing. Surprisingly, tNGS only detected MTB in 3 of 15 samples. The sensitivity of tNGS detection using FFPE specimens was only 20%, which was much lower than using fresh biopsied specimens.

## 4 | Discussion

Reports using the technology of NGS whether mNGS or tNGS for ITB diagnosis and CD-LTBI discrimination are scarce. The diagnostic values of NGS for TB affecting different organs are inconsistently reported, which cannot be directly applied to ITB. The sensitivity (46%–84%) of mNGS in PTB detection was either similar to or slightly better than other traditional tests in different reports, which hinders the prioritisation of mNGS in the diagnosis of PTB [11, 15–17]. However, mNGS using cerebrospinal fluid had significantly higher sensitivity than traditional diagnostic methods in diagnosing tuberculosis meningitis and was highly recommended in clinical practice [11]. In this study, 100 cases with undetermined ulcers and positive IGRA who could not be clearly diagnosed as CD-LTBI or ITB were included in the analysis. TNGS showed optimal sensitivity and specificity in ITB detection. The performance of tNGS in ITB diagnosis, considering sensitivity, specificity, NPV and PPV, was similar to the outcomes of a report examining tuberculosis meningitis and

**TABLE 2** | Diagnostic performances of AFB, TB-PCR, caseous necrotising granuloma and tNGS for ITB.

| Test | AFB | TB-PCR | Caseous necrotising granuloma | tNGS |
|---|---|---|---|---|
| Sensitivity (95% CI) | 15 (4–39) | 22 (12–36) | 17 (9–28) | 83 (72–91) |
| Specificity (95% CI) | 100 (60–100) | 100 (81–100) | 100 (81–100) | 100 (87–100) |
| PPV (95% CI) | 100 (31–100) | 100 (68–100) | 100 (68–100) | 100 (92–100) |
| NPV (95% CI) | 32 (16–54) | 35 (23–48) | 28 (18–39) | 76 (60–87) |

Abbreviations: AFB, acid-fast bacillus; CI, confidence interval; ITB, intestinal tuberculosis; TB-PCR, tuberculosis polymerase chain reaction; tNGS, targeted next-generation sequencing.

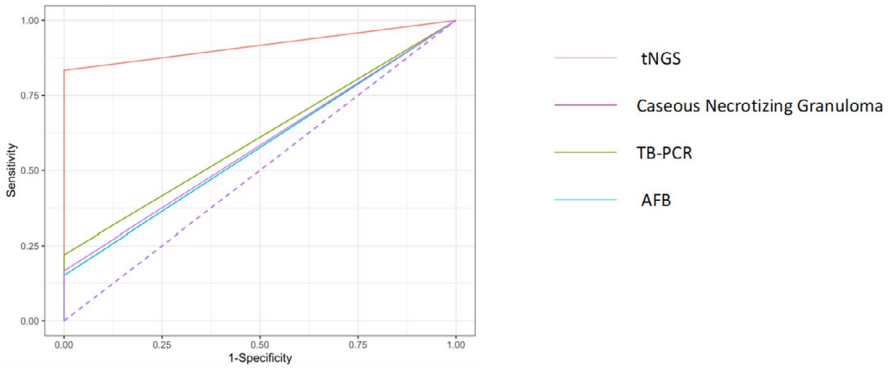


**FIGURE 2** | ROC curves comparing the diagnostic performances of tNGS, AFB, TB-PCR and caseous necrotising granuloma. AFB, acid-fast bacillus; TB-PCR, tuberculosis polymerase chain reaction; tNGS, target next-generation sequencing.

**TABLE 3** | Comparison of four diagnostic models for ITB diagnosis.

| | Model 1 | Model 2 | Model 3 | Model 4 |
|---|---|---|---|---|
| AUC | 0.98 | 0.98 | 0.98 | 0.98 |
| Sensitivity | 0.97 | 0.97 | 0.97 | 0.97 |
| Specificity | 0.91 | 0.91 | 0.91 | 0.91 |
| PPV | 0.95 | 0.95 | 0.96 | 0.96 |
| NPV | 0.94 | 0.94 | 0.94 | 0.94 |

*Note:* Model 1 included tNGS, CRP and large granuloma. Model 2 included tNGS, CRP, large granuloma and AFB. Model 3 included tNGS, CRP, large granuloma and caseous necrotising granuloma. Model 4 included tNGS, CRP, large granuloma, AFB and caseous necrotising granuloma.
Abbreviations: AUC, areas under the curves; ITB, intestinal tuberculosis; NPV, negative predictive value; PPV, positive predictive value.

better than average outcomes for PTB [11]. In addition, none of the patients with CD-LTBI was positive in tNGS. Hence, tNGS is valuable for the rapid diagnosis of ITB and its discrimination from CD-LTBI.

Moreover, tNGS outperformed the other three methods in terms of sensitivity. As most positive findings from the other three methods have been already covered by tNGS, the combination of tNGS with other methods is of little value. The diagnostic rates obtained by AFB staining and detection of caseous necrotising granuloma were within the normal ranges and consistent with previous records [4, 9]. Nevertheless, the diagnostic potential of the TB-PCR method was lower than the averages of outcomes in previous reports showing an average sensitivity of 47% (42%–51%) and a specificity of 95% (93%–97%) [8]. The outcome of the TB-PCR method varies significantly in different reports, possibly due to different primer choices or distinct commercial kits [18]. The low abundance of tubercle bacilli in isolated ITB cases, the use of paraffin-embedded samples for TB-PCR and the sensitivity of primers might have contributed to the variation observed in this study. Importantly, tNGS has the advantage of detecting microorganisms of low abundance, which are undetectable using traditional methods such as TB-PCR. The amount of bacteria in extrapulmonary TB samples before suppurative changes was below the detection threshold of conventional molecular tests [17]. When considering traditional methods for comparison with tNGS, TB culture was finally omitted in this study. MTB grows very slowly, and traditional solid culture methods (e.g., the Roche culture method) usually take 4–6 weeks to produce results [14]. Although culture is still considered one of the gold standards for diagnosing TB, it provides limited support for rapid diagnosis and medication guidance. Physicians often initiate ATT without waiting for culture results. Thus, the use of culture for ITB diagnosis is becoming increasingly rare.

Furthermore, nearly all conventional microbial tests detect only one or a limited panel of pathogens at a time, while tNGS simultaneously identifies a broad range of pathogens [19]. Compared to TB-PCR, tNGS provides more consistent and reliable results, as tNGS detected two cases with CMV infection from suspected ITB, hence avoiding empirical ATT for these two cases. Lastly, in this study, tNGS identified ITB within 24 h. Meanwhile, the other three methods took more than 3 days to yield results, consistent with previous literature [9, 20]. Compared with mNGS, tNGS also offers several advantages. It is not affected by the human genome and background flora, as the detection target specifically focuses on preselected pathogenic pathogens, excluding human gene fragments as well as the gene fragments of background flora [21]. Besides, tNGS testing takes less turnaround time and costs less ($100 per tNGS test vs. $300 per mNGS test). Lastly, tNGS testing is currently commercially available in China (as in the USA, western Europe and India [20]), serving as a rapid and accurate diagnostic approach [22]. Thus, tNGS is a cost-effective option that can be used to distinguish ITB from CD-LTBI.

The appropriate choice of specimens for tNGS testing was crucial for improving diagnostic performance. Detection of ITB using specimens acquired from less invasive techniques, such as blood and stool collection, has been scarcely reported previously. Detecting bacterial colonisation in stool is complex due to disturbance by multiple factors, resulting in a low detection rate [23]. In addition, previous studies utilising stool samples for TB diagnosis showed great inconsistency [23]. The detection of free intestinal colonising flora in the blood is rare; therefore, the use of blood for tNGS to detect ITB has not been reported. Lung biopsy tissues have been used for the detection of PTB, showing better diagnostic performance than bronchoalveolar lavage fluid [24]. To date, the use of intestinal biopsy tissue for tNGS has not been reported in the literature. In a previous report using a fluorescent DNA probe for MTB detection, 24 positive cases out of 35 total cases (68.5%) were obtained from necrotising granulomatous inflammatory sites [7]. Therefore, in this study, NGS

was performed using biopsy tissue samples taken from the ulcer bases, where necrotising granulomatous inflammatory sites are likely located. However, the detection efficiency of 15 ITB cases in the preliminary trial was low. The number and activity of MTB would have been decreased in the examined samples upon paraffin embedding. Therefore, fresh biopsy tissue samples were selected for this study, as the sample collection process is readily available.

Still, 17% of patients with ITB had negative tNGS results, leading to difficulty in rapid discrimination of ITB from CD with concomitant LTBI. As a result, empirical treatment with ATT still needed to be initiated. However, there is no single index to differentiate these two diseases with both specificity and sensitivity over 80%, let alone exceeding the diagnostic yield of the current mucosal tNGS. A novel diagnostic nomogram including seven variables to differentiate between ITB and CD still had a sensitivity of about 80%, but this diagnostic tool is not clinically practical to use [25]. Furthermore, this nomogram was not validated for the differentiation of ITB and CD with concomitant LTBI. We have also established models combining different diagnostic methods and clinical characteristics. Incorporating factors like CRP and large granuloma (Model 1) could improve sensitivity (97% vs. 83%) and especially NPV (94% vs. 76%) compared to tNGS alone. However, single indicator like tNGS is more convenient and simple to use compared to models in clinical practice. Furthermore, as shown in Table 3, the addition of the other three diagnostic methods does not offer significantly more advantages over tNGS alone.

Because the IGRA is not 100% specific, the inclusion of patients with positive IGRA results may have led to a very few ITB cases being missed in the real-world setting. Previous studies have shown that factors such as HIV infection, low peripheral lymphocyte count, advanced age and prior usage of immunosuppressants are associated with false-negative IGRA results [26]. Repeated testing may help reduce omissions. Whether tNGS confers diagnostic benefits to these patients demands further investigation.

In conclusion, tNGS using fresh biopsy tissue samples from ulcer bases is highly sensitive and specific in diagnosing ITB and shows superiority over other traditional diagnostic methods. TNGS might be used as a frontline diagnostic method for ITB diagnosis and an efficient method to discriminate from CD with LTBI, particularly in high TB-endemic countries.

### Author Contributions

**Lingna Ye:** conceptualization, investigation, writing – original draft, validation, project administration, writing – review and editing, methodology, data curation, supervision, formal analysis, visualization. **Yushu Cao:** investigation, writing – original draft, formal analysis, data curation, conceptualization. **Yujuan Fu:** investigation, project administration, methodology, formal analysis, data curation. **Chuwen Tian:** investigation, methodology, data curation, formal analysis. **Qian Cao:** supervision, project administration, conceptualization, formal analysis.

### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

The data and materials used in this study are not publicly available due to ethical restriction. Requests should be directed to the corresponding author.

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**Supporting Information**

Additional supporting information can be found online in the Supporting Information section.